PKA-dependent ENaC trafficking requires the SNARE-binding protein complexin

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Submitted 3 November 2003; accepted in final form 13 June 2005

The ENaC holochannel is composed of three homologous subunits; α, β, and γ (18, 19, 21). ENaC exhibits characteristic biophysical properties of low single-channel conductance (~5 pS), long open and closed times, a high-sodium ion (Na⁺)-to-potassium ion (K⁺) selectivity ratio (>100:1), and is blocked by the diuretic amiloride at submicromolar concentrations (30). The synthesis, expression, and activity of ENaC are regulated by numerous factors including long (aldosterone)- and short (vasopressin, insulin)-term hormonal regulation, external and internal sodium ion (Na⁺) concentrations, regulation by fluid flow, membrane stretch, protease activity, and interactions with other proteins and transporters (20, 41).

In many ENaC-expressing tissues and model cells, a rapid stimulation of sodium transport is elicited by the action of vasopressin, which increases intracellular CAMP levels to activate protein kinase A (PKA) (3, 13, 14, 20, 48). This stimulation of ENaC activity can be brought about by an increase in open probability (Pₒ) or by an increase in the number of channels resident in the apical membrane, mechanisms that need not be mutually exclusive (44). Several studies have suggested that the acute increase in Na⁺ transport is the result of regulated insertion of ENaC-containing membrane vesicles into the apical plasma membrane, thus increasing the cell surface pool of transporting channels (5, 6, 15, 38). The residence time for channels at the apical surface is also regulated by removal and degradation of ENaC, which are mediated by binding of the ubiquitin ligase Nedd4–2 to the cytoplasmic COOH termini of ENaC subunits. All three subunits contain a proline-rich region (PPXYYXL) which interacts with Nedd4 to target ENaC for ubiquitin-dependent internalization and degradation (2, 16, 22, 36, 50). Mutations in the P-Y motifs at the COOH termini of ENaC result in an increased plasma membrane channel number and corresponding upregulation of Na⁺ transport (2, 22, 50).

The mechanisms by which ENaC is delivered to the membrane are less clearly defined. The soluble N-ethyl-maleimide-sensitive factor attachment protein receptor (SNARE) family of membrane traffic, regulatory, and fusion proteins is well characterized for their role in neurotransmitter (NT) release and their involvement in regulated exocytosis (9, 12, 17, 47, 49). Different SNAREs are localized to donor and acceptor membranes where they form a high-affinity complex to promote membrane fusion. Vesicle-associated (v)-SNAREs are located on cargo-containing intracellular vesicles (e.g., synaptobrevin/VAMP) which assemble with cognate target membrane (t)-SNAREs (syntaxin and syntaxosomal-associated...
protein of 25/23 kDa or SNAP-25/23), allowing the subsequent assembly of trans-SNARE complexes between the vesicle and plasma membrane to facilitate vesicle fusion (7, 56). Several accessory proteins have been described, which facilitate both the assembly and disassembly of SNARE complexes. Primary among these are complexin, which stabilizes the SNARE complex (see below) and the ATPase NSF (N-ethylmaleimide-sensitive factor) and α-SNAP (soluble NSF attachment protein), which disassemble SNARE complexes after membrane fusion to release SNAREs for recruitment in future trafficking events (49).

SNAREs have been implicated in ENaC regulation in both a direct and indirect manner. Coexpression of syntaxin 1A with ENaC in Xenopus laevis oocytes resulted in a marked reduction in ENaC currents (11, 42, 46). This inhibition was mediated via direct physical interaction of the H3 domain (SNARE motif) of syntaxin with ENaC subunit COOH termini. This inhibition was reversed when either the H3 region was deleted or the COOH termini of ENaC subunits were truncated (10, 42). The syntaxin-ENaC interaction suggests a role for syntaxin 1A in regulated insertion of ENaC-containing vesicles at the apical membrane.

A novel SNARE-complex binding protein, complexin/synaphin, has been described to function in Ca^{2+}-mediated NT release in neurons (25, 37). Complexin binds only to assembled trans-SNARE complexes, composed of syntaxin, VAMP, and SNAP25/23 in the groove between the syntaxin and VAMP helices, but it does not bind significantly to individual unassembled SNARE components (39, 55). Complexin’s role in neurons appears to involve the stabilization of assembled trimeric SNARE complexes to facilitate NT release. It was recently reported that either overexpression or silencing of complexin expression in pancreatic β-cells resulted in impaired glucose-induced β-cell insulin secretion (1). These findings implicated complexin in regulated exocytic events and demonstrated that an optimal expression level of complexin may be required for exocytosis.

The potential role of complexin in SNARE-mediated ENaC insertion into the apical membrane was investigated in this study. No role for complexin in epithelial tissues has been reported previously, and the expression pattern of complexin in the kidney has not been examined. We show that the complexin II isoform is expressed in the mouse kidney by cDNA screening. Coexpression of complexin and ENaC in X. laevis oocytes resulted in a marked inhibition of ENaC currents. The effect of overexpression or knockout of complexin on acute ENaC regulation was examined in a mouse cortical collecting duct (mCCD) cell line. Increased or decreased complexin expression inhibited ENaC currents, indicating that an optimal level of complexin expression is required for both basal and stimulated ENaC activity. Surface biotinylation demonstrated that the complexin-induced reduction in short-circuit current ($I_{sc}$) was due to a reduction in apical membrane-resident ENaC, and the lack of cAMP stimulation resulted from the inability of complexin-overexpressing cells to insert additional ENaC into the apical membrane. Immunofluorescent localization of SNARE proteins expressed at the apical domain of the mCCD epithelia identified candidates for the cognate SNARE interactions that mediate ENaC insertion, which have been shown to be stabilized by complexin. These findings further reinforce the role of channel insertion as a means of short-term regulation of Na$^+$ absorption and demonstrate that a complexin-stabilized SNARE trafficking machinery is required for this process.

**MATERIALS AND METHODS**

**Antibodies and reagents.** For immunofluorescent labeling, the following primary antibodies were used: rabbit anti-syntaxin 1 (1 mg/ml) and rabbit anti-syntaxin 3 (0.3 mg/ml) polyclonal, anti-zona occludens 1 (ZO-1) rat monoclonal (Chemicon, Temecula, CA), anti-synaptobrevin 2 (VAMP2) mouse monoclonal (1 mg/ml), anti-SNAP-23 rabbit polyclonal (1 mg/ml), and anti-complexin I/II (1 mg/ml) rabbit polyclonal (Synaptic Systems, Gottingen, Germany). All secondary antibodies raised against the relevant species (rat, mouse, or rabbit) were obtained from Molecular Probes (Eugene, OR), conjugated to Alexa Fluor-488 or -568 and highly cross-absorbed (all at a stock concentration of 1 mg/ml). For ENaC biotinylation and Western blot analysis, a rabbit polyclonal antibody was raised against an epitope on the extracellular loop of α-ENaC corresponding to the human amino acid sequence 103-201 (RYYPEIKEEELDIREQTG). This antibody, along with polyclonal antibodies recognizing β-ENaC (kindly provided by M. Knepper, Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD) or γ-ENaC (Abcam, Cambridge, MA), has been previously characterized and used in similar studies (5). Forskolin (10 mM stock), 8-bromoadenosine-3′,5′-cyclic monophosphate (8-Br-cAMP, 100 mM stock), and (Arg9)-vasopressin (100 IU/ml) were obtained from Sigma (St. Louis, MO).

cDNA murine kidney screen and complexin cRNA production. Complexin II cDNA was amplified from a murine kidney cDNA library (Clontech, Palo Alto, CA) using the following oligonucleotides specific for the complexin II sequence 5′-TAACCAAGGCCTGCAGATG-3′; 3′-GGGATGGGTTACTTCTTGAAC-5′ (54). PCR was performed using PFU Taq polymerase (Invitrogen, Carlsbad, CA) following the manufacturer’s protocol. A PCR product of ~600 bp was cloned into the pcBluntII-TOPO vector (Invitrogen). A complexin II-containing clone was isolated and verified by sequencing. For mCCD cell transfection (see below), the complexin II sense (sns) and antisense (as) constructs were subcloned into the pcDNA3.1-mEGFP and pcDNA3.1-mENaC subunits (kindly provided by Dr. T. Kleyman, Renal-Electrolyte Division, Department of Medicine, University of Pittsburgh, PA), and complexin II were synthesized using T3 or SP6 mMessage mMachine (Ambion, Austin, TX).

Oocyte cRNA injection and two-electrode voltage-clamp recordings. Oocyte isolation and RNA injection were performed as described previously (52). Briefly, 1 ng of cRNA of each ENaC subunit was injected into stage V or VI oocytes. For experiments investigating the effect of complexin coexpression on ENaC function, oocytes were coinjected with 1 ng of complexin II cRNA in addition to 1 ng of each ENaC subunit. Expression proceeded at 18°C for 16–24 h in sodium-free ND96 solution before ENaC current recordings. Two-electrode voltage-clamp recordings were performed as described previously (52) using 3 M KCl-filled micropipettes connected to a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA). Oocytes were bathed continuously in ND96 solution as follows (in mM): 96 NaCl, 1 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, and 5 HEPES, pH 7.4. In sodium-free ND96, equimolar N-methyl-d-glucamine chloride replaced NaCl. After impalement, membrane potentials were allowed to stabilize before voltage clamping at ~100 mV; amiloride-sensitive Na$^+$ currents were recorded as the difference in current before and after addition of 10 μM amiloride at the same ~100-mV holding potential. To record current-voltage relationships, oocytes were clamped from −140 to +60 mV in increments of 20 mV for 500 ms at each holding potential. The individual number of recordings (n) is presented along with the total number of animals from which the oocytes were obtained (N).

**Cell culture.** The mpkCCD$_{14}$ cells (kindly provided by A. Vandevelle and M. Bens of the Institut National de la Santé et de la
Recherche Médicale, Paris, France) were grown in flasks (passages 30–40) in defined medium as described previously (57). Growth medium was composed of equal volumes DMEM and Ham’s F12 supplemented with 60 nM sodium selenate, 5 μg/ml transferrin, 2 mM glutamine, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 20 mM d-glucose, 2% vol/vol FBS, and 20 mM HEPES (Invitrogen GIBCO and Sigma), pH 7.4 at 37°C in 5% CO₂–95% air atmosphere. The medium was changed every second day. For all experiments, the mCCD cells were subcultured onto permeable filter supports (0.4-μm pore size, 0.33- or 45-cm² surface area, Transwell, Corning Costar, Cambridge, MA). Cells were kept on filters for at least 7 days in defined medium which was changed every second day. Typically, after 7 days, a confluent transporting cell monolayer had developed which could be assessed by recording open-circuit voltage (Voc) and transepithelial resistance (Rt) measurements using “chopstick” electrodes (Millipore, Bedford, MA). Polarized transporting monolayers exhibited typical Voc values >40 mV and Rt > 1,500 Ω·cm². At least 24 h before use in any investigation, medium incubating cells on filters was replaced with a minimal medium (without drugs or hormones) which contained only DMEM, Ham’s F12, HEPES, and glucose.

mCCD cell transfection. Cells were plated onto 35-mm Petri dishes and grown to ~90% confluency and transfected with complexin sense and antisense pcDNA 3.1 Hygro+ plasmids constructs (see above) or GFP-actin (Clontech) as a control, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s recommendations. After 12 h, transfection medium was replaced with full medium (as described above) containing 200 μg/ml hygromycin B (Invitrogen) for complexin or 400 μg/ml Geneticin/G418 Sulfate (Mediatech, Herdon, VA) for GFP-actin, to select for cells which had incorporated the plasmids. Stable heterogeneous populations of hygromycin (hygro)- or G418-resistive cells were obtained after 2 wk of selection. Control, untransfected cells all died in either hygro- or G418-containing media during the same period. Cultures were maintained in selective media until subcloned onto filter supports, at which point they were returned to full media (without hygro or G418) until 24 h before use, wherein they were incubated in minimal medium as described. A loss of complexin phenotype was observed when the stable cell lines were passaged over 10 times, and a second stable line was produced to ensure a reproducible phenotype was maintained. In all studies, cell populations were never passaged more than five times to ensure consistency in complexin phenotype and expression. Results from both stable line presentations are shown with the various stable lines described as mCCDsn1 and mCCDsn2 for the two complexin sense lines, both stable lines are presented with the various stable lines defined as mCCDas for the antisense line, mCCDactin for GFP-actin cell line and mCCDwt for the control, untransfected wild-type cells.

Immunoprecipitation, Western blot analysis, and surface biotinylation. The immunoprecipitation and the Western blotting protocol were performed as described previously (5, 32). Protein assays (BCA, Pierce Biotechnology, Rockford, IL) ensured equivalent amounts of protein were used for Western blot analysis and immunoprecipitation. Detection of complexin II in mCCD cells was accomplished by collecting cells grown on filters in lysis buffer (0.4% deoxycholate acid, 1% NP-40, 50 mM EDTA, 10 mM Tris-HCl at pH 7.4) and immunoprecipitating 200 μg of mCCDwt or mCCDsn1 cell lysate using the complexin-specific antibody (Synaptic Systems). The precipitated product was resolved on a 12% SDS-PAGE gel and transferred to nitrocellulose. The membrane was then subjected to Western blot analysis to identify complexin II. Labeling specificity was tested by incubating the anti-complexin antibody with an equal concentration of the immunizing peptide (Synaptic Systems) for 2 h at 37°C before use in Western blotting. Mouse brain lysate (5 μg) was used as a positive control. Surface biotinylation was performed as previously described (5, 31). Cells grown on 75-mm-diameter inserts (Transwell, Corning Costar) were divided into two groups and either stimulated with 10 μM forskolin 30 min before biotinylation or remained untreated in growth medium at 37°C. The inserts were then washed five times with ice-cold PBS containing Mg²⁺ and Ca²⁺ (Cambrex BioScience, Walkersville, MD) to remove medium containing FBS. The cells were biotinylated in borate buffer (85 mM NaCl, 4 mM KCl, 15 mM Na₂HPO₄, 375 μg biotin at pH 9) on the apical surface with the basolateral side of the monolayer bathed in medium containing FBS to prevent basolateral biotinylation. After 20 min, basolateral and apical sides were aspirated and medium containing FBS was placed on the cells to quench the signal. Monolayers were washed five times with ice-cold PBS with agitation, and the cells were harvested. Cell homogenate was obtained by lysing cells in lysis buffer (see above) and then centrifuged for 5 min at 5,000 rpm. Cell homogenate was assayed for protein concentration, and 300 μg of protein were incubated with 150 μl avidin bead slurry as previously described (58). Samples were heated to 100°C for 8 min and separated on a 10% SDS-PAGE. Samples were transferred to nitrocellulose membranes (Millipore) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA) following the manufacturer’s protocol. Nitrocellulose was blocked in 5% skim milk constituted in PBS for 3 h. The membrane was transferred to 1% skim milk–PBS containing antibodies (1:500), for β-ENaC, 1:200; α-ENaC, 1:1,000) at 4°C overnight. Following antibody, incubation blots were washed four times in PBS. Horseradish peroxidase-conjugated secondary antibodies (KPL, Gaithersburg, MD) were diluted 1:5,000 in 1% skim milk–PBS. Membranes were incubated with secondary antibody for 1 h at room temperature. The membrane was washed twice for 30 s in PBS followed by one 15-min wash and four 5-min washes. Reactive proteins were visualized with enhanced chemiluminescence (PerkinElmer Life Sciences, Wellesley, MA).

Short-circuit current recordings. Cells cultured on filter supports were mounted in modified, closed Costar Using chambers, and the cultures were continuously short circuited (0 mV) with an automatic voltage clamp (Department of Bioengineering, University of Iowa, Iowa City, IA). Transepithelial resistance was measured by periodically applying a 2.5-mV bipolar pulse and calculated using Ohm’s law. The bathing Ringer solution was composed of (in mM) 120 NaCl, 25 NaHCO₃, 3.3 KH₂PO₄, 0.8 K₂HPO₄, 1.2 MgCl₂, 1.2 CaCl₂, and 10 d-glucose. Chambers were constantly gassed with a mixture of 95% O₂–5% CO₂ at 37°C which maintained the pH at 7.4. At the end of each experiment, 10 μM amiloride was added to the apical cell surface to determine the net Na⁺ transport through ENaC.

Immunofluorescent labeling. Cells cultured on filters or coverslips were prepared for microscopy in the same manner. Cells were washed three times in PBS at 4°C (to be referred to as PBS wash) to remove media. For apical membrane counterstaining Alexa Fluor-633 (Molecular Probes)–conjugated wheat germ agglutinin (WGA) was added to the apical solution diluted 1:200 in PBS (stock solution 1 mg/ml in dH₂O, 2.9 mol dye/mol). Cells were washed in cold PBS and fixed in 1% paraformaldehyde (pH 7.4 in PBS) for 20 min at 4°C. After PBS wash, apical cell membranes were permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 15 min. Following a PBS wash, cells were incubated in blocking media comprising 10% skim milk and 10% normal goat serum (Amersham Biosciences, Piscataway, NJ) in PBS at 4°C for 20 min. Primary antibodies were diluted in PBS containing 10% skim milk, and the cells were incubated for 12 h after another PBS wash. Primary antibodies were diluted as follows: for anti-VAMP2 and anti-SNAP23 1:500, for anti-syntaxin 1 and 3 1:200, for anti-complexin and anti-ZO-1 1:100. For peptide inhibition controls, antibodies were incubated in the presence of twice the concentration of the immunizing peptide at 37°C for 30 min before incubation with the cells. For labeling including an anti-ZO-1 antibody counterstain, rat monoclonal primary antibodies were coincubated with rabbit polyclonal antibodies at 4°C for 12 h. Cells were PBS washed following primary antibody labeling, and relevant secondary antibodies in PBS with 10% skim milk were added to the apical surface of the inserts and incubated for 12 h at 4°C at a dilution of 1:500. After a final PBS wash, cell nuclei were stained with a combination of Hoechst 33258 (Sigma) diluted 1:5,000 and either TO-PRO3 (Molec-
Oocyte coexpression of complexin reduces ENaC currents. A mouse kidney cDNA library was screened for the expression of complexin by making use of specific primers (as described in MATERIALS AND METHODS). The presence of the mouse complexin by making use of specific primers (as described in MATERIALS AND METHODS). The expression of complexin in mCCDns1 cells was lower than that in mouse kidney cDNA in the forward and reverse directions (sense and antisense), the levels of complexin protein expressed in these cells with only linear adjustments to brightness and contrast performed on nondeconvoluted images. Orthogonal xy-images were obtained from the reconstructed three-dimensional (3-D) images (presented beneath confocal images). To determine the localization of some of the SNARE proteins more accurately, blind image deconvolution was performed on the data stacks using Autodeblur (Autoquant, Albany, NY), which automatically obtained point spread functions from the raw data volume. Deconvolved optical sections were then reconstructed using Imaris software (Bitplane, St. Paul, MN) to produce a 3-D image.

Statistics. Data are presented as means ± SE with comparisons between groups performed by simple Student’s t-test analysis, and significant values were considered at \( P < 0.05 \) (SigmaStat, SPSS, Chicago, IL).

RESULTS

Oocyte coexpression of complexin reduces ENaC currents. A mouse kidney cDNA library was screened for the expression of complexin by making use of specific primers (as described in MATERIALS AND METHODS). The presence of the mouse complexin II isoform in the kidney was confirmed by sequence analysis of the PCR product. The expression of complexin in the kidney was consistent with a previous report which used Northern blot tissue screening to detect small amounts of complexin mRNA in the rat kidney and lung with more substantial expression in the brain and testis (26).

The effect of complexin overexpression on ENaC function was assessed by coinjection of complexin and ENaC cRNAs into \( X. \) laevis oocytes. Oocytes were injected with 1 ng each of mouse \( \alpha \)-, \( \beta \)-, and \( \gamma \)-ENaC cRNA with and without 1 ng mouse complexin II mRNA. The effect of complexin on expressed ENaC currents is illustrated in Fig. 1. The average amiloride-sensitive current for control oocytes expressing ENaC subunits alone was 4,363 ± 876 nA (\( N = 4 \), \( n = 16 \)) from all recordings (Fig. 1A). When ENaC was expressed with complexin II, the \( \mathrm{Na}^+ \) current was significantly reduced to an average of 584 ± 143 nA (\( P < 0.0003 \), \( N = 4 \), \( n = 19 \)). To account for small deviations in expressed currents from different batches of oocytes, the currents were normalized to the average value for control ENaC expressing oocytes in the same experiment from the same individual (Fig. 1B). Normalized currents from complexin-coexpressing oocytes were reduced to 16 ± 3% of the control current. The current-voltage relationship for ENaC-expressing oocytes in the presence or absence of complexin is presented in Fig. 1C. The reversal potential of ~0 mV for all conditions indicates that the oocytes were \( \mathrm{Na}^+ \) loaded; however, the minimal rectification and sensitivity to amiloride demonstrate that the recorded currents were most likely due to ENaC-mediated \( \mathrm{Na}^+ \) transport (\( N = 3 \), \( n = 9 \)).

Complexin expression in mCCD epithelia. To determine whether alterations in the levels of complexin influenced the regulation of ENaC in a renal cell line that endogenously expresses ENaC, three stably expressing mCCD lines were established, in which complexin was constitutively over- or underexpressed. By constructing plasmids encoding complexin cDNA in the forward and reverse directions (sense and antisense), the levels of complexin protein expressed in these cells were enhanced or markedly reduced, as demonstrated in Western blots obtained following immunoprecipitation of complexin from cells cultured on filters (Fig. 2A). The expression of complexin in mCCDns1 cells was lower than that in mouse brain lysate, which was used as a positive control and complexin was undetectable in the mCCDns cell line. These experiments were repeated with similar results (\( n = 2 \)). The available complexin antibody was unable to distinguish be-

Fig. 1. Whole cell currents from 2 electrode voltage-clamp recordings in oocytes. A: amiloride-sensitive current from oocytes expressing \( \alpha \)-, \( \beta \)-, and \( \gamma \)-ENaC alone (filled bar) or ENaC and complexin (open bar); \( N \) = number of individual animals, \( n \) = total number of oocytes. Bars represent mean values ± SE. B: amiloride-sensitive currents from \( \alpha \)-, \( \beta \)-, and \( \gamma \)-ENaC plus complexin-expressing oocytes (open bar) normalized to oocytes expressing ENaC alone (filled bar). C: current-voltage relationships for \( \alpha \)-, \( \beta \)-, and \( \gamma \)-ENaC (triangles) or ENaC plus complexin expressing oocytes (circles) in the presence or absence of amiloride (see inset). Oocytes were clamped from −140 to 60 mV in increments of 20 mV for 500 ms to obtain steady-state current values (\( N = 3 \), \( n = 9 \) for each point).
between the two highly homologous isoforms and would therefore recognize both complexin isoforms in the mCCD cells. It is clear in the mCCDa line that expression of complexin was reduced as no signal was detected. In addition, preincubation of the complexin antibody with the immunizing peptide reduced detectable signal in the mCCDsns1 cells, demonstrating the specificity of the antibody for the complexin epitope. The immunofluorescent localization of complexin in mCCDsns1 cells (Fig. 2B) displayed an expected cytosolic distribution for this soluble protein. No detectable signal by immunofluorescence was observed in the mCCDa cell line where complexin expression was reduced (data not shown).

Complexin expression reduces $I_{sc}$. Basal $I_{sc}$ measurements taken from cells cultured on filter inserts mounted in Ussing chambers (Fig. 3A) were consistent with results from oocyte recordings, as unstimulated amiloride-sensitive currents were significantly reduced compared with mCCDwt. In addition, there was no significant response to forskolin stimulation in either of the mCCDsns1 and mCCDsns2 ($P = 0.34$) or mCCDas cell lines ($P = 0.31$).

In the control mCCDwt- and mCCDactin-expressing cell lines, forskolin stimulation resulted in an increase in $I_{sc}$ to a level almost double that of the basal prestimulated values after 25 min, and no significant difference was observed in the responses of these control lines. This indicated that the transfection and selection procedure did not affect ENaC transport properties. The addition of 10 μM amiloride at the end of each experiment ($t = 40$ min) demonstrated that ENaC $Na^+$ transport accounted for more than 90% of recorded $I_{sc}$. In contrast to control epithelia, all three complexin-transfected cell lines had significantly reduced basal $I_{sc}$ which could not be stimulated significantly by the addition of forskolin (Fig. 3A), vasopressin (data not shown), or 8-bromo-cAMP (Fig. 3B).

Two complexin-overexpressing stable cell lines were established to demonstrate that the inhibition of ENaC currents in the CCD cells was due to an effect of complexin overexpression. The selected cells were not clonal populations, and the
mixed cell lines gave similar results. For both mCCDsns$_1$ and mCCDsns$_2$ lines, basal and stimulated ENaC currents were reduced and there was no significant difference in $I_{sc}$ recordings between these stable cell lines. Both lines exhibited significant reductions in basal and stimulated $I_{sc}$ compared with control mCCDwt and mCCDact.

**ENaC surface labeling.** The reduction in $I_{sc}$ in the mCCDsns lines could be due to either a decrease in channel $P_o$ or a reduction in channel number at the apical surface. To address the possibility that $I_{sc}$ in mCCDsns cells was reduced due to diminished apical membrane expression, surface biotinylation was performed on mCCDwt and mCCDsns cell lines to determine the amount of apical membrane-resident ENaC under control and stimulated conditions. The specificity of the α-ENaC antibody used has been demonstrated previously by preincubation with the immunizing peptide (5). As a control, actin was not biotinylated, ensuring that only extracellular labeling was performed (data not shown) (5). Densitometric quantitation of three separate biotinylation experiments is summarized in Fig. 4. The surface expression of all three ENaC subunits under basal, unstimulated conditions was significantly reduced in the mCCDsns$_1$-overexpressing cells. In addition, there was no significant increase in any of the surface-labeled ENaC subunits following 30 min of forskolin stimulation. Previous studies (5) and the results presented in Fig. 4 show an increase in all three labeled ENaC subunits following forskolin stimulation. The use of confocal microscopy and image deconvolution, the localization of syntaxins 1 and 3 in single cells could be resolved further. Figure 6, A and B, represents a reconstruction of immunolabeled syntaxin 1 and 3 in single cells from a cultured mCCD monolayer. Counterstains for the nuclei and ZO-1 help to orientate the relative position of syntaxins 1 and 3 within these cells. It is clear that the majority of the immunofluorescence detected was located above the nucleus, capping the cells, at the apical pole. Additional colocalization studies would be required to determine the exact localization of the syntaxin isoforms. However, little signal was detected beneath the level of the tight junctions as determined by ZO-1 counterstaining.

**DISCUSSION**

The effects of the SNARE-binding protein complexin on ENaC regulation are reported in this study. The complexin II isoform was identified in a mouse kidney library and was expressed specifically in mCCD epithelial cells. Coexpression of complexin cRNA with mouse ENaC subunits in oocytes resulted in a reduction in ENaC current. Mouse CCD cells overexpressing complexin or cells with decreased complexin expression exhibited reduced basal ENaC currents and failed to respond to cAMP stimulation. These data are in agreement with a recent report in which glucose-stimulated insulin secretion from pancreatic β-cells was disrupted when complexin expression was increased or knocked down (1).

Vesicle trafficking has been characterized extensively in neuronal cells in the release of NT and other signaling molecules (9, 12, 17, 29, 51). Specific SNARE proteins coordinate the docking and fusion of vesicles with their target membranes to deliver their contents in a rapid, cyclical, and site-specific manner. There is evidence that similar mechanisms are in operation within epithelial cells (33, 35). Both target membrane t-SNARE and vesicle-associated (v)-SNARE proteins assemble at the exocytic interface to stabilize the apposition (docking) of donor and acceptor membranes so that fusion can occur (9). The specific role of complexin in NT release remains poorly understood; nevertheless, overexpression of either complexin isoform was thought to bind only to assembled SNARE complexes, the presence of relevant apically targeted SNARE proteins in mCCD cells had to be established. Immunofluorescent staining of syntaxins 1 and 3, VAMP 2, and SNAP-23 in polarized mCCDwt cells cultured on permeable supports is presented in Fig. 5, A–D. The apical polarization of the two syntaxin isoforms makes these likely t-SNARE candidates which would associate with cytoplasmic vesicles containing ENaC and the v-SNARE, VAMP2. The specificity of the syntaxin antibodies was verified by preincubation of the syntaxin antibodies with the relevant immunizing peptide. It is clear from Fig. 5, E and F, that the majority of the detectable signal was eliminated by preincubation with their specific immunizing peptides. Immunofluorescent localization of syntaxins 2 and 4 produced a weaker basolateral staining pattern (data not shown); a similar localization of these isoforms has been noted in Madin-Darby canine kidney cells and rat kidney (33). VAMP2 had a more cytoplasmic localization with a punctate staining pattern, consistent with its localization in intracellular vesicles. By making use of confocal microscopy and image deconvolution, the localization of syntaxins 1 and 3 in single cells could be resolved further. Figure 6, A and B, represents a reconstruction of immunolabeled syntaxin 1 and 3 in single cells from a cultured mCCD monolayer. Counterstains for the nuclei and ZO-1 help to orientate the relative position of syntaxins 1 and 3 within these cells. It is clear that the majority of the immunofluorescence detected was located above the nucleus, capping the cells, at the apical pole. Additional colocalization studies would be required to determine the exact localization of the syntaxin isoforms. However, little signal was detected beneath the level of the tight junctions as determined by ZO-1 counterstaining.

**SNARE protein expression in mCCD.** As complexin is associated with cytoplasmic vesicles containing ENaC, and the v-SNARE, VAMP2, the specificity of the syntaxin antibodies was verified by preincubation of the syntaxin antibodies with the relevant immunizing peptide. It is clear from Fig. 5, E and F, that the majority of the detectable signal was eliminated by preincubation with their specific immunizing peptides. Immunofluorescent localization of syntaxins 2 and 4 produced a weaker basolateral staining pattern (data not shown); a similar localization of these isoforms has been noted in Madin-Darby canine kidney cells and rat kidney (33). VAMP2 had a more cytoplasmic localization with a punctate staining pattern, consistent with its localization in intracellular vesicles. By making use of confocal microscopy and image deconvolution, the localization of syntaxins 1 and 3 in single cells could be resolved further. Figure 6, A and B, represents a reconstruction of immunolabeled syntaxin 1 and 3 in single cells from a cultured mCCD monolayer. Counterstains for the nuclei and ZO-1 help to orientate the relative position of syntaxins 1 and 3 within these cells. It is clear that the majority of the immunofluorescence detected was located above the nucleus, capping the cells, at the apical pole. Additional colocalization studies would be required to determine the exact localization of the syntaxin isoforms. However, little signal was detected beneath the level of the tight junctions as determined by ZO-1 counterstaining.
plexin isoform in PC12 cells markedly suppressed NT release (27).

Despite the uncertainty about the precise function of this cytosolic protein, several important features have been described. First, complexin exhibits a specificity of binding only to assembled, four-helix SNARE complexes. No interaction has been observed between complexin and individual SNARE proteins (39). 3-D NMR analysis of the complexin-SNARE interaction indicated that complexin specifically stabilized the VAMP and syntaxin interface by binding in the groove formed between the \( \alpha \)/H9251-helices of these two proteins once the core complex was assembled (8). Second, complexin was previously found to compete with \( \alpha \)/H9251-SNAP for SNARE-complex binding (28, 37, 55). As \( \alpha \)/H9251-SNAP, along with NSF, is responsible for the disassembly of SNARE complexes, complexin's competitive binding may well disrupt the steady-state availability of free SNAREs. The individual knockout of either of the two complexin isoforms in mice produced mild phenotypes associated with neuronal defects, but no observations in these single knockouts reported a role for complexin in other tissues (24, 43, 53). It was suggested that the remaining isoform of complexin would be capable of substituting for the deleted protein as the two are structurally very similar. In keeping with this, double complexin knockout mice die shortly after birth, mainly due to impaired Ca\(^{2+} \)/H11001-dependent NT release (43).

Reports from studies which demonstrated an inhibition of ENaC currents in response to overexpression of SNAREs, in particular syntaxin 1A, defined a role for SNARE proteins in ENaC regulation and more specifically in regulated trafficking of ENaC (10, 42, 46). The effect of syntaxin overexpression on ENaC appears to involve two actions, a direct but minor

Fig. 5. Immunofluorescent localization of SNARE proteins in mCCD cells. The localization of VAMP2 (A), SNAP-23 (B), and syntaxin 1 and syntaxin 3 (C and D) are presented with an xy-optical section taken at the apical domain (arrow) above the corresponding xz-image. Note the apical localization for the t-SNAREs (B-D) with a more diffuse and punctate intracellular staining of VAMP2 in A. White bars = 5 \( \mu \)m; yellow arrows on the xz-image depict the level of the corresponding xy-image. The apical membrane in A and B is specifically labeled with WGA (see MATERIALS AND METHODS) and colored blue for orientation. Peptide competition controls for anti-syntaxin 1 (E) and 3 (F) antibodies. Epifluorescent images were taken with identical settings for exposure time and camera gain settings with no postcapture alterations to contrast or brightness. White bars = 10 \( \mu \)m.

Fig. 6. Three-dimensional reconstruction following image deconvolution of single cells labeled for syntaxin 1 (A) and 3 (B). Localization of syntaxin 1 (green in A) is clearly above the level of the tight junctions (ZO-1 colored red) capping the nucleus (gray). A similar localization is observed for syntaxin 3 labeling (B). White bars = 25 \( \mu \)m; degree of axial rotation of the xy-plane presented in the top right of each image.
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reduction in current by modulation of channel gating, and a more significant disruption of ENaC delivery to the plasma membrane (11). Overexpression of syntaxin 1A may reduce ENaC currents by disrupting the stoichiometric SNARE interactions required for ENaC trafficking, whereas optimal physiological levels of this t-SNARE are required for coordinated insertion events. If SNARE proteins are being employed to target ENaC-containing vesicles to the apical membrane, any alterations in not only the proteins themselves but also in other SNARE-binding proteins could result in altered ENaC regulation. As complexin competes with α-SNAP for binding to SNARE complexes (37), the possibility exists that overexpression of complexin would disrupt the regulated disassociation of assembled trans-SNARE complexes by NSF and α-SNAP and interfere with the turnover of essential SNARE components needed for vesicle docking and fusion.

In contrast to the dual mode of inhibition of ENaC surface number and \( P_O \) by syntaxin overexpression, inhibition of ENaC activity by complexin would be a direct result of trafficking events, as this protein has been reported to bind only to SNARE proteins once an assembled complex has been formed. Complexin was described specifically to target and stabilize assembled SNARE complexes to promote vesicle fusion and in the case of neuronal cells, more rapid NT release (4, 37, 39, 40, 43).

Coexpression of ENaC with complexin in oocytes produced an ENaC current inhibition of over 80% compared with control currents (Fig. 1). The same phenomenon was observed when complexin was overexpressed in mCCD cells. In the case of epithelial cells, the effect of overexpression of complexin could also be tested under basal and stimulated conditions. The acute response to forskolin, observed in control cells, was more significant disruption of ENaC delivery to the plasma membrane. The reduction of ENaC density at the apical surface of cells overexpressing complexin along with the corresponding reduction in Na\(^+\) currents suggest that complexin is involved in the regulated delivery and expression of ENaC at the apical surface in mammalian CCD cells.

ACKNOWLEDGMENTS

We appreciate the gift of an anti-syntaxin 3 antibody (T. Weimbs and S.-H. Low, Lerner Research Institute, Cleveland, OH) used in the preliminary studies for this work. We thank C. Rice and C. King for invaluable technical assistance.

GRANTS

This work was supported by a postdoctoral fellowship grant from the Cystic Fibrosis Foundation and Training Grant DK-061296–03 (M. Butterworth) and National Institutes of Health Grants RO1-DK-54814 (to R. Frizzell) and R01-DK-057718 (to J. Johnson).

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